Early intervention in the 3xTg-AD mice with an Aβ-antibody fragment ameliorates first hallmarks of Alzheimer disease

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Short Title: ScFv-h3D6 ameliorates AD hallmarks in 3xTg-AD mice

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Abbreviations used: AD, Alzheimer disease; BPSD, behavioral and psychological symptoms of dementia; MWM, Morris water maze; scFv, single chain variable Fragment.

ABSTRACT

The single-chain variable fragment, scFv-h3D6, has been shown to prevent in vitro toxicity induced by the Aβ peptide in neuroblastoma cell-cultures by withdrawing Aβ oligomers from the amyloid pathway. Present study examined the in vivo effects of scFv-h3D6 in the triple-transgenic 3xTg-AD mouse model of Alzheimer disease. Prior to the treatment, five-month-old female animals, corresponding to early stages of the disease, showed the first BPSD-like behaviors as assessed in the MWM. These behaviors included long- and short-term learning and memory deficits and high swimming navigation speed. After a single intraperitoneal dose of scFv-h3D6 the swimming pattern was reversed to normal levels and the learning and memory deficits were ameliorated. Brain tissues of these animals revealed global decrease of Aβ oligomers in the cortex and olfactory bulb after treatment, but this was not seen in the hippocampus and cerebellum. In the untreated 3xTg-AD animals we described an increase of both apoJ and apoE concentrations in the cortex, as well as an increase of apoE in the hippocampus. Interestingly, treatment significantly recovered the non-pathological levels of these apolipoproteins. In conclusion, our results suggest that the benefit of scFv-h3D6 occurs at both behavioral and molecular levels.

Keywords: Alzheimer disease, ScFv, immunotherapy, behavior, Aβ oligomers, apoE, apoJ, clusterin.

INTRODUCTION

Amyloid β immunotherapy has shown promises in the treatment of Alzheimer Disease (AD), as indicated by the implementation of ongoing set of clinical trials[1]. The effectiveness of active immunization with Aβ-peptide in removing amyloid plaques, was clearly shown in the noted AN-1792 clinical trial[2]. Although the occurrence of neuroinflammatory complications halted this trial[3], follow-up of the immunized patients revealed slowing down of cognitive decline, even one year after administration of the last doses[4]. The main drawback of active immunotherapy is the inability to intervene once immune system is activated; this makes passive immunotherapy (administering Aβ-directed antibodies) an attractive alternative.

Humanized murine monoclonal antibody AAB-001 (mAb-h3D6), or Bapineuzumab, which targets the N-terminal 1–5 residues of the Aβ peptide, is being tested in several clinical trials[5]. In Phase II trials, besides vasogenic edema, side effects of Bapineuzumab were mostly mild and transient. The observed edema was thought to be dose-related and more likely to occur in *APOE4* carriers. However, in subsequent Phase III trials, which avoided high doses of bapineuzumab and recruited only *APOE4* non-carriers, results obtained were still disappointing hence the trial was stopped in August 2012[6].

In order to avoid meningoencephalitis, vasogenic edema and micro-cerebral hemorrhages which can occur with the use of complete antibodies, the use of single-chain variable fragments (scFv); antibodies devoid of Fc portion and incapable of directly activating microglia, is becoming an attractive therapeutic strategy[7-9]. We previously described how to obtain recombinant scFv-h3D6, a single chain variable fragment derived from mAb-h3D6[5, 10, 11], which precludes cytotoxicity of $A\beta_{1-42}$-oligomers

in SH-SY5Y neuroblastoma cell-culture, by withdrawing them from the amyloid pathway[12]. Since evidence suggests that therapeutic interventions that reduce Aβ fibrils at the cost of augmenting non-fibrillar Aβ assemblies, including Aβ oligomers, could be harmful[13] targeting Aβ-oligomers, rather than amyloid plaques, is highly recommended[14].

Our results from cell-culture studies of scFv-h3D6 treatment led us to investigate its disease prevention potential in an in vivo model of AD. The triple-transgenic mouse for Alzheimer disease (3xTg-AD) harboring $APP_{Swe,}$ $PS1_{M146V}$, and tau_{P301L} transgenes, represents a unique animal model, which mimics both amyloid and tau AD neuropathologies in an age-dependent manner and in disease-relevant brain regions[15]. At early stages of the disease, immunohistochemical presence of intraneuronal Aβ can be detected in the cortex, hippocampus and amygdale with this demonstrable at 4 months of age[15-17]. At these early stages of the disease the model mimics the first "behavioral and psychological symptoms of dementia (BPSD)" which are followed by impairment of cognition[18-21]. By 6 months of age, studies have reported the presence of deficits at the electrophysiological, synaptic and cholinergic levels as well.

The present work aimed to study the effects of intraperitoneal administration of scFv-h3D6 in 3xTg-AD females on some behavioral and molecular aspects at early stages of Alzheimer disease.

RESULTS

BPSD-like symptoms before the treatment. As detailed in Table 1, the corner and the open-field tests evidenced that, at 5 months of age, the first BPSD-like symptoms were already apparent in the 3xTg-AD mice. Thus, in the corner test, these mice exhibited an anxious-like flight behavior as measured by increased number of corner visits [Student *t*-test, $p<0.05$]. In the open-field test, the same animals exhibited freezing behavior (latency of initial movement) and impaired thigmotaxis (latency to leave the center and to reach the periphery). As illustrated in Fig. 1, reduced exploratory activity was observed at different times of the test and in the total accumulated counts in both horizontal and vertical locomotor activities (Fig. 1A and B, respectively). No differences were found in the other variables.

Assessment of behavioral effects of the treatment. Fig. 2 illustrates the results obtained 'day by day' and 'trial by trial' in the different tasks performed in the Morris water maze (MWM) after the treatment. Escape latency (panel A) shows that all the animals spent the same time to reach the visual platform [V1: G, T and GxT, all $F_{(1,26)}$ >0.395, *n.s.*] with a clear improvement through the trials [V11 to V14: t, RMA, $F_{(1,26)}$=8.506, $p<0.001$]. On the next day, namely PT1, the platform was hidden and changed to a reversal position which was maintained until the end of the task. There, a genotype effect [PT1: G, $F_{(1,26)}$ =4,023, $p<0.05$] indicates a difference in long-term memory in the 3xTg-AD groups as compared to NTg animals, which is mostly seen between the untreated 3xTg-AD and the treated NTg animals [post-hoc Duncan's test, $p<0.05$]. The detailed 'trial by trial' analysis indicated that scFvh3D6 was also able to improve the performance of the NTg animals on PT11, the first trial of the place learning task [post-hoc Duncan's test, $p<0.05$] since the animals insisted to look for the platform in the prior location [Pearson correlation, r=.905**, $p<0.01$]. In contrast, the

untreated NTg animals and the two 3xTg-AD groups spent the same amount of time as that in their first experience in the maze. Still, a generalized progress with time was seen in this [PT1: t, $F(2,52)=6.875$, $p<0.01$] and the following days [PT2: t, $F(2,52)=3.090$, $p<0.05$; PT3: t, $F(2,52)=7.422$, $p<0.001$]. On the last day and, overall, during the three days of the place learning 'time x genotype x treatment' [PT3: txGxT, $F(2,52)=3.309$, $p<0.05$; PT1 to PT3: txGxT, RMA, $F(2,52)=3.624$, $p<0.05$] and 'time x genotype' [PT1 to PT3: txG, $F(2,52)=3.190$, $p<0.05$] interaction effects were observed which indicate genetic differences in the progress of the short and long-term learning and memory processes and that the effects of the treatment were dependent on the genotype.

Interestingly, when the trajectories covered during the tasks were analyzed consistent genotype differences were found in the navigation speed in all the tasks recorded. The results are presented as distance covered to reach the platform (Fig. 2B) and swimming speed (Fig. 2C).The 'day by day' analysis [V, PT1, PT2, PT3 and PT1 to PT3, G, all F's$(1,26)>5.270$, $p<0.05$] indicated an overall higher swimming speed in the 3xTg-AD animals as compared to the NTg genotype. Besides, in the place learning, a time effect [t, $F(2,52)=10.212$, $p<0.01$] was found. Most importantly, treatment effects were found in the 'day by day' analysis of both MWM tasks [V: T, $F(1,26)=7.397$, $p<0.01$; PT1 to PT3: $F(1,26)=5.280$, $p<0.05$; PT1: $F(1,26)=13.794$, $p<0.001$] with a reduction of the speed in scFv-h3D6 treated animals. Moreover, treatment x genotype interaction effects on cue learning [V: GxT, $F(1,26)=9.820$, $p<0.01$] and the last day of place task [PT3: GxT, $F(1,26)=4.638$, $p<0.05$] indicated selective treatment effects on the 3xTg-AD genotype, where scFv-h3D6 reduced the high swimming speed to the normal values shown by NTg animals. In most of these cases, the post-hoc Duncan's test revealed that the untreated 3xTg-AD mice differed from the other three experimental groups of mice [$p<0.05$]. The distance revealed that the main difference

was found on the first day of place learning [PT11: F(3,26)=4.079, p<0.05, all the other trials, F's (3,26)>0.091, *n.s.*] where the untreated 3xTg-AD mice covered a significant higher distance to find the platform as compared to all the other groups of mice [post-hoc Duncan's test, p<0.05]. The results also showed that on the last trial of the place task, untreated 3xTg-AD mice also differed from the untreated NTg mice [PT33: F(3,26)=0.132, p<0.05; post-hoc Duncan's test, p<0.05] and that this genotype difference was lost after treatment with scFv-h3D6.

On the probe trial (see Fig. 3), preference for the trained quadrant versus the other ones was measured. The computerized analysis of the trajectories indicated that the untreated 3xTg-AD mice showed an increased swimming speed (Fig. 3A) as compared to untreated NTg mice [Student *t*-test, t=-2,936, p<0.05] and this was found reversed to normal levels in the scFv-h3D6-treated 3xTg-AD animals [Student *t*-test, t=1.984, p<0.05 vs 3xTg-AD, and *n.s.* vs both NTg groups]. Due to the differences in the speed, the standard measure of permanence (Fig. 3C) was complemented by the measure of annulus crossings (Fig. 3B) and distance covered (Fig. 3D). All the groups showed a similar number of entries to the exact location of the platform in the previous test. The NTg group slightly discriminated the trained quadrant albeit did not reached the statistical significance. However, the factorial analysis showed genotype x treatment interaction effects on both the percentage of time spent and the distance covered in the opposite quadrant [Distance in the O: F(1,26)=4.220, p<0.05; Time spent in the O: F(1,26)=8.052, p<0.01; all the others, F's (1,26)<2.934, *n.s.*]. The results indicated differences between the untreated 3xTg-AD and NTg groups of mice, with 3xTg-AD mice spending a higher time and covering a longer distance in the opposite quadrant as compared to the NTg counterparts [post-hoc Duncan's test, p<0.05 vs NTg mice, in both cases]. The within subjects paired *t*-test analysis indicated that in both 3xTg-AD groups the opposed quadrant was preferred, instead of the trained one. However, the

treatment with scFv-h3D6 reduced the significance of this preference for the wrong quadrant [the statistical significance was reduced from $p<0.001$ to $p<0.05$]. These results were in agreement with the search strategies shown (Fig. 4 and Table 2). That is, 'scanning' and 'random swimming' were the most frequent strategies in all the groups while 'wall hugging' and 'flotation' were only observed in the untreated NTg and 3xTg-AD mice, respectively. 'Direct swims' and 'focal searching' were more characteristic of NTg animals and scarcely seen in the groups of 3xTg-AD mice. The treatment improved the search strategies in both genotypes, in the case of 3xTg-AD by reducing the non-goal directed 'chaining' while in the NTg mice the benefit was obtained by increasing 'direct swims'.

Effect of scFv-h3D6 in the Aβ-aggregation profile in vivo. Mice subjected to the behavioral studies were subsequently sacrificed and several brain subregions, hippocampus, cortex, olfactory bulb and cerebellum, dissected to prepare protein extracts of each group as described in the Materials and Methods Section. Fig. 5 depicts an illustrative immunoblotting analysis with 6E10 antibody of the soluble Aβ-amyloid oligomers from extracellular extracts (TBS fraction). Medium-molecular-mass assemblies (>10kDa) that might represent Aβ oligomers were observed. This species pattern were unaltered when the initial SDS-PAGE did not contain 8M urea (not shown), suggesting that these species are held together by urea-resistant hydrogen bonding. In agreement with other studies[9, 22, 23], multiples of trimeric Aβ oligomers were detected, i.e. dodecamers, nonamers, hexamers and trimers. Additionally, sAPPα (secreted form of APP by α-secretase cleavage) was also detected.

Aβ-aggregation profiles for NTg mice did not change upon scFv-h3D6 treatment. Mouse sAPPα, dodecamers, nonamers, and hexamers of Aβ-peptide were detected in all of the samples, being the profile characteristic of the studied brain subregion. It is

essential to note that other studies have also detected these mouse species with 6E10 antibody[22]. Similar species were seen in the 3xTg-AD mice; although human sAPPα has higher mobility than mouse sAPPα and the relative abundance of each oligomer was dramatically increased. In fact, since mouse oligomers are being detected in the NTg mice, this evident increase is due to the oligomers being processed from human sAPPα.

As expected, human sAPPα was not detected in the cerebellum, and scFv-h3D6 treatment did not change the profile pattern of the oligomeric species in this area. In the hippocampus, human sAPPα was clearly detected, although the profile pattern of the oligomeric species upon scFv-h3D6 treatment did not change. In contrast, the treatment changed the aggregation profile of the Aβ-peptide in the cortex and in the olfactory bulb. Both cortex and olfactory bulb showed a dramatic decrease in dodecamers, nonamers, and hexamers levels (Fig. 5). It was also likely that the trimer detected in the untreated cortex disappeared upon scFv-h3D6 treatment.

Since the intracellular Aβ has been proposed to be responsible for the first behavioral symptoms observed in 3xTg-AD transgenic mice[15, 18, 19, 24], the intracellular protein (1% Triton fraction) was analyzed by immunoblotting. No significant change in the profiles was found between treated and non-treated groups (Fig. 6). Mainly α-APP and trimeric Aβ were detected in the hippocampus; whereas the band for dodecamers was rather tiny and those for nonamers and hexamers were not detected. In the cortex only α-APP was detected, whereas in olfactory bulb and cortex any band was observed. Similarly, in the membrane-associated protein (2% SDS fraction) only a faint band for α-APP and a clear band for the nonamer were detected in the hippocampus, and in the rest of the areas just the nonamer was present (not shown). Finally, the formic acid extracts did not show any result (not shown). On the other hand, NTg groups rendered similar

results with both the intracellular and membrane-associated protein extracts (not shown).

Effect of scFv-h3D6 in recruiting clusterin (APOJ) and APOE. Because there is evidence of the in vivo involvement of clusterin (apoJ) and apoE on Aβ amyloid fibril formation[25, 26], we quantified by ELISA their content in the extracellular TBS fraction (Fig. 7). Clusterin concentration was strongly increased in the cortex of 3xTg-AD when compared to NTg animals (Fig. 7A, 2.8 fold). ScFv-h3D6 treatment of 3xTg-AD mice significantly decreased this level and there were no significant difference between the NTg groups (treated or untreated) and the treated 3xTg-AD, indicating that the treatment restored the non-pathological level of clusterin in the cortex. The levels of clusterin in hippocampus, olfactory bulb, and cerebellum did not significantly differ upon treatment.

ApoE concentration was increased in hippocampus (1.7 folds) and cortex (1.8 folds) of 3xTg-AD mice when compared to NTg animals, and scFv treatment clearly restored the non-pathological levels in both areas (Fig. 7B). The level of apoE in olfactory bulb and cerebellum did not show significant differences.

DISCUSSION

We recently showed that an antibody fragment, the single-chain variable fragment scFv-h3D6, has the ability to prevent the toxicity induced by the Aβ peptide in human neuroblastoma cell-cultures[12]. In the present work, the scFv-h3D6 in vivo effects was studied in the triple-transgenic 3xTg-AD mouse model of Alzheimer disease at 5 month of age, which corresponds to early stages of the disease[15]. The therapeutical potential of a single i.p. dose of 85 µg scFv-h3D6 was assessed in females since (i) this route of administration enables an easy translation to humans, and (ii) females have been reported to better exhibit the cognitive and BPSD-like symptoms of the disease[16, 19-21].

ScFv-h3D6 treatment improved cognition and reversed BPSD-like symptoms.

Before the treatment, we confirmed the presence of BPSD-like symptoms[19, 27] and determined the level of behavioral disturbances of 3xTg-AD animals at early stages of the disease as compared to NTg mice. In agreement with our previous reports[19-21, 27], anxious-like behavior was observed in the corner test, were the novelty of the home-cage induced an increased number of corner visits and rearings as a fight-or-flight copying with stress strategy. With the progression of the disease or under more anxiogenic experimental conditions, like those of the open-field test, this neophobia is expressed as freezing and reduced activity[19]. Thus, in the subsequent behavioral test, neophobia to the white and illuminated open arena was expressed as an initial freezing behavior, which was, among all, the most sensitive variable to show the genetic differences between 3xTg-AD and the NTg mice. The initial fear was followed by an extra delay in all the sequence of behavioral events that usually occur in this test: the exit of the centre to reach the periphery, the start of vertical exploratory activity and the self-grooming behavior which starts as soon as the main exploratory activity slows down. A clear reduction of horizontal and vertical components of locomotor activity was

also observed. Thereafter, these results confirming the presence of behavioral disturbances were also used as criteria to allocate, for each genotype, the mice in the two treatment groups in a counterbalanced manner[28].

In order to assess the effects of scFv-h3D6 on cognition and, indirectly, on some BPSD-like symptoms, animals were submitted to a series of paradigms in the MWM[29]. The tests started with a process of acquisition in the cue learning task which is useful as a first experience in the maze in fearful animals[19]. The performance was optimal in all the groups, with the intramaze cue facilitating the localization of the platform. The lack of genotype differences in this task allowed us to discard the possible confounds of motivational or sensoriomotor differences. All the animals were able to achieve the same level of post-habituation baseline at V14 which emphasized the subsequent results. In the first trial of the place task, the hidden platform and the unexpected change in the usual platform position introduced a certain degree of difficulty to the test. This is shown by the animal's insistence on looking for the platform in the previous location, and thus needing more time and covering more distance, before finding the new position as compared to the previous sessions. Therefore, the genotype effect found in this first trial of the place task was indicative of differences in long–term memory and worse behavioral flexibility in the 3xTg-AD mice. The results were reinforced by the longer distance covered to find the platform, both at the beginning and the end of the place learning task, only shown by the untreated 3xTg-AD mice while the 3xTg-AD animals treated with scFv-h3D6 showed a normal behavior.

Another finding at the behavioral level was the ability of the scFv-h3D6 treatment to reverse the increased swimming speed shown by 3xTg-AD animals into a normalized pattern. This hyperactive swimming pattern was previously characterized[20] and it is considered a common BPSD-like symptom in this animal model since these animals also show hyperactivity in non-anxiogenic conditions[19], including increased voluntary

running activity in wheels[21]. Therefore, among all, the effectiveness of scFv-h3D6 on swimming speed provided the most striking finding because it was a consistent phenomenon all through the different trials of the test. Moreover, this is the first time that a treatment modality completely reverses this behavioral hallmark of 3xTg-AD mice, unlike other cases where treatment success modifying swimming speed was not as clearly consistent and effective[20, 21]. It is also interesting to note that the benefits of scFv-h3D6 were extended to the NTg mice, which may suggest the putative benefits of scFv-h3D6 in the normal population as well. In this sense, AD-purposed treatments targeting Aβ processing have also been shown to act as cognitive enhancers in NTg mice (i.e.[28]). The time effect in the latency and the distance in both the cue and place learning tasks is mostly related to short and long-term hippocampal-dependent learning processes. In contrast, the time effect in the navigation speed could be understood as a result of a more goal-directed navigation ability, thanks to the prior experiences[20]. Interestingly, the increased swimming speed and the search strategies observed in the 3xTg-AD mice in the removal test confirmed the observations obtained in the previous paradigms. Altogether, these results reinforce the fact that behavioral pattern of 3xTg-AD at 5 month of age is already disrupted, as shown by the presence of BPSD-like symptoms and poorest cognitive performance. This included observed increased swimming speed and cognitive impairments mostly affecting long-term memory, although some short-term alterations could be observed. The present results suggest that scFv-h3D6 can be a promising therapeutic strategy and further studies are needed to extent these data.

ScFv-h3D6 treatment eliminated Aβ-oligomers.

It is now widely accepted that soluble Aβ-oligomers are the cause of loss of synapses and the neuronal injury, characteristic of Alzheimer disease[14, 30]. This hypothesis

explains the lack of correlation between cognitive decline and the presence of amyloid plaques[31]. Therefore, it makes sense to develop strategies to promote their elimination, a fact that is supported by the finding that therapeutic interventions that reduce Aβ fibrils at the cost of augmenting Aβ oligomers could be harmful[13]. ScFv-h3D6 derives from mAb-h3D6[12], or Bapineuzumab, and specifically recognizes Aβ oligomers[10].

In order to compare the aggregation extent of the Aβ peptide in different brain areas of treated and non-treated animals, protein extracts from hippocampus, cortex, olfactory bulb and cerebellum were analyzed by western-blot analysis with the 6E10 antibody. Twelve, nine and six-mer oligomers of the Aβ peptide were globally decreased in cortex and olfactory bulb upon treatment, but this effect was observed neither in the hippocampus nor in the cerebellum. The different sensitivity observed in the cortex and olfactory bulb is in agreement with the neuroanatomical progression of the affectation of the brain in the human patient, starting at the enthorrinal cortex. The olfactory bulb, which is mostly cholinergic, is also one of the most sensitive areas. Similar results have been also obtained in our colony of animals, with cortex being the best area of study at early stages of the disease[32].

Despite the fact that 6E10 antibody should theoretically detect all forms Aβ, we only detected αAPPα and multiples of trimeric Aβ oligomers (dodecamers, nonamers, hexamers and trimers). This could be due to the presence of low molecular weight species (mainly monomers and dimers) being outside the lower limit of detection of our methodology or to their total absence. Interestingly, the oligomeric species detected in the extracellular extracts, which were decreased upon treatment as mentioned above, are those that have been related to the pathology of the disease in the literature. Dodecamers and nonamers promoted cognitive decline when intracranially injected in the Tg2576 mouse model of AD[22], whereas hexamers and trimers where decreased upon DNA vaccination of the 3xTg-AD mouse model[23]. Therefore, scFv-h3D6 is

effective in eliminating the most toxic species of Aβ-oligomers and constitutes a promising therapy.

A part from the controversy raised with the report claiming that the intraneuronal material share epitopes with full-length APP but not free Aβ[33], there is abundant evidence documenting the occurrence of intraneuronal Aβ in the normal and diseased human brain[34]. In any case, it is not clear whether the impairment of neural function is due to the extracellular fraction or intracellular fraction or to both. LaFerla *et al*. correlated synapses and memory loss with the amount of intracellular Aβ[35]. Precisely in the 3xTg-AD model, removal of extracellular Aβ plaques is immediately followed by the clearance of intraneuronal Aβ, indicating that a dynamic equilibrium exists between the two pools[36]. The fact that in the present work the effect of immunotherapy was observed on the extracellular pool and not in the intracellular one has been also reported in other cases where fractionation with detergents is applied (i.e[9, 22, 34]. Moreover, there are contradictory results showing a decrease in the intracellular pool concomitant to a increase in the extracellular one upon immunotherapy[9]. Also, some groups state that intracellular Aβ is not an indicator of cognitive decline neither in AD nor DS because its level is maintained during the progression of both diseases[37]. In any case, AD dementia strongly correlates with soluble Aβ level (i.e.[31]), which is clearly diminished in the cortex and olfactory bulb of five-month-old 3xTg-AD females upon scFv-h3D6 treatment.

ScFv-h3D6 treatment restored clusterin and apoE levels.

In this work we found increased levels of clusterin and apoE in the cortex of 3xTg-AD animals, together with an increased level of apoE in hippocampus, and, more interestingly, the restoration to non-pathological levels upon scFv-h3D6 treatment. The

different sensitivity observed in the cortex and hippocampus is in agreement with the neuroanatomical progression of the affectation of the brain in the 3xTG-AD mouse[38].

Apart from being a lipid-transporter, clusterin (encoded by the *CLU* gene), also known as apolipoprotein J (apoJ), is an ATP-independent extracellular chaperone, mainly secreted by astrocytes, that has been found in association with all extracellular deposits of protein aggregates associated with diseases that have been studied so far[39], including those produced in AD[25, 26]. It is known that clusterin inhibits the formation of aggregates by binding to their surface hydrophobic-residues[40] and that β oligomers of different sizes (from dimers to 50-mers) interact with clusterin to form stable complexes[26]. These complexes are removed by interaction with megalin (LRP-2)[41, 42] and other receptors of the LDLR family[43]. Since exposition of hydrophobic residues of the Aβ:ScFv-h3D6 complex has been reported[12], it is tempting to propose that clusterin could have internalized the complex and, as a consequence, both clusterin and Aβ-oligomers would be decreased in the extracellular fraction of treated animals.

There is strong evidence that clusterin and apoE are regulated in a cooperative manner (DeMattos et al., 2004). Non-neuronal cell types, most notably astroglia and microglia, are the primary producers of the lipid transporter apoE, while neurons preferentially express apoE receptors. ApoE is found to colocalize with amyloid-β and plays a role in enhancing its clearance and degradation[44, 45]. In fact, the human *APOE4* allele increases the risk of developing late-onset AD plausibly because it binds Aβ with approximately 20-fold lesser avidity than the more abundant allele *APOE3*[46]. APOE4-specific changes in Aβ accumulation have also been recently described in EFAD-Tg mice.[47] On the other hand, haploinsufficiency of human *APOE* reduces amyloid deposition in a mouse model of Aβ amyloidosis and results in significantly decreased amyloid plaque deposition and microglial activation.[48], suggesting a relationship

between inflammation and apoE. From our results, it is likely that the opposite is also true: targeting Aβ-oligomers with scFv-h3D6 has diminished them and, as a consequence, apoE levels have been restored. This is of importance, because, although further studies with scFv-h3D6 are required, neuroinflammation was not likely to occur as a side effect. This would be in agreement with other studies claiming that immunotherapy with scFv molecules is safer than that with the complete antibodies[7-9].

The suggested benefits of scFv-h3D6 at the behavioral level, its capability for eliminating Aβ oligomers and to restore clusterin and apoE levels in the 3xTg-AD model of Alzheimer disease encourages us to further investigate its therapeutic potential.

MATERIALS AND METHODS

Animals

Triple-transgenic 3xTg-AD mice harboring PS1/$_{M146V,}$ APP$_{Swe}$ and tau$_{P301L}$ transgenes were genetically engineered at the University of California Irvine, as previously described[15]. Briefly, two independent transgenes (encoding human APPSwe and human tauP301L, both under control of the mouse Thy1.2 regulatory element) were co-injected into single-cell embryos harvested from homozygous mutant PS1M146V knock-in (PS1KI) mice. Since the PS1 knock-in mice were originally generated in a hybrid 129/C57BL6, this hybrid was used as the control strain,

Thirty 5-month-old females animals from the Spanish colony of homozygous 3xTg-AD and wild-type non-transgenic (NTg) mice (*n*=15 each group), established in the Medical Psychology Unit, Autonomous University of Barcelona[27] were used in the present study. Three to five animals of the same genotype and sex were maintained (Makrolon, 35 x 35 x 25 cm) under standard laboratory conditions (food and water *ad lib*, 22 $\pm$ 2 ºC, 12 h light: dark cycle starting at 08:00).

Production of scFv-h3D6

ScFv-h3D6 was recombinantly expressed in *E. coli* and purified as previously described[12]. It is important to note that lipopolysaccharides (LPS), the major endotoxins of gram-negative bacteria, were removed from the protein by using Detoxi-Gel Endotoxin Removing columns (Thermo Scientific).

Experimental design

Animals were assessed in the corner (CT) and open-field (OF) tests in order to determine the level of behavioral disturbances shown by 3xTg-AD mice before the treatment and as compared to age- and gender-matched NTg mice[19]. Thereafter, mice

were allocated in two treatment groups counterbalanced on the basis of the mean locomotor activity recorded in these tests[28]).

Animals were treated with a single intraperitoneal dose of 85 μg scFv-h3D6 or vehicle (PBS-buffer). Twenty-four hours after the administration, the animals were assessed during 4 consecutive days for cognition and BPSD-like behaviors in a series of MWM paradigms (MWM)[19]. Behavior was evaluated by both direct observation and analysis of videotape recorded images by an observer unaware of the animals' genotype and treatment. Experiments were performed under dim white-light conditions (16-20 lux) from 10:00 to 13:00 and in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Council Directive (86/609/EEC Council) on this subject.

Behavioral assessment prior to the treatment

Corner test

Neophobia to a new home-cage was evaluated in the corner test for 30 seconds. Animals were individually placed in the center of a clean standard home-cage (Makrolon, 35 x 35 x 25cm) filled with wood shave bedding. Number of corner visits, latency to realize the first rearing, and the number of rearings were recorded.

Open-field test

Immediately after the corner test, mice were placed in the centre of the apparatus (home-made, wooden, white, 55x55x25 cm high) and observed for 5 min. Horizontal (crossings of 5 x 5 cm squares) and vertical (rearings) locomotor activities were recorded for each minute of the test. We also recorded the latency of the sequence of the following behavioral events: initial freezing (latency of initial movement), thigmotaxis or discrimination of unprotected/protected areas in the test (latency of leaving the central 5 x 5 cm square and that of entering the peripheral ring 5cm to the walls).

Emotional behaviors such as self-grooming behavior (latency, number, and duration of groomings), number of defecations and presence/absence of urine were also measured.

Assessment of behavioral effects of the treatment

Morris water maze tests

The effects of the treatment on the cognitive abilities of the animals were assessed in three paradigms for learning and memory in the Morris water maze (MWM)[29] consisting of one day of cue learning with a visual platform (V), three days of place task for long-term and short-term spatial reference memory with a hidden platform (PT) followed, after removal of the platform (RM), by one probe trial for short-term memory. The mice were trained to locate a platform (7 cm diameter) in a circular pool (Intex Recreation Corp., Calif., USA; 91cm diameter, 40 cm height, 25ºC opaque water) located in a black test room with distal cues. Animals failing to escape within 90 s were manually guided to the platform. In the trials with platform, all animals stayed on it for 5–10 s before being removed. Immediately after, the animals were dried and then placed in a holding cage with a heating pad to prevent hypothermia.

Day 1, Cue learning with a visual platform

On the first day, animals were trained to criterion (90% escaping under 60s). This required four visible platform trials (V1-V4) in a single day. The last visible platform trial of any animal is considered to be its post-habituation baseline, and was designated V4 (visible platform trial 4). Intertrial interval was 30 minutes. The visible platform remained in the same place for all of the trials and was outlined by a black and white stripped flag with high contrast to the white background made by the addition of white, non-toxic tempera paint. Animals were delivered to either the west or east quadrant (the target quadrant being designated as north) in a random manner for each trial as these are

equidistant from the target. High contrast visual cues were placed on the wall of the pool in each quadrant.

Day 2 to day 4, Place learning with a hidden platform

Twenty-four hours after the last visible platform trial, the animals were tested in a series of three hidden platform trials (PT1–PT3). Animals were delivered to either the west or east quadrant and the target quadrant being designated as south, that is, reversal to the location in the previous paradigm. As before, the trials were staggered in time to ensure a stable inter-trial interval of about 30 min and each trial was a maximum of 90 s long.

Day 4, Removal

In order to measure the retention and level of accuracy of the precise location of the platform position achieved, the animals were allowed to swim in the probe trial or 'removal' 2 h after the end of the last session of the place task. The procedure consisted of removing the platform from the maze and releasing the mouse from the north starting point and letting the animal navigate for 60s.

During the trials of the learning tasks, the escape latency was measured with a stop-watch. In all trials, the trajectories were recorded and analyzed using the SMART system 2.5.14 (Panlab, S.L., Barcelona, Spain) in order to measure the total distance covered and to calculate the swimming speed. In the probe trial, the trajectories were analyzed to determine the number of annulus crossings, the time spent in each quadrant of the pool, as well as the incidence of non-goal directed (floating, wall hugging, chaining and random swimming) and goal-directed (scanning, focal searching and direct searching) search strategies[49].

Protein extraction

Animals were sacrificed at the end of the behavioral tests, which is 5 days after administration of scFv-h3D6. The extraction was prepared by sequential centrifugation of brain subregions homogenates as previously described[22]. Frozen tissues of hippocampus, cortex, olfactory bulb and cerebellum from 5-month-old 3xTg-AD and NTg mice used in the experiment were weighted and mechanically homogenized in ice-cold TBS supplemented with protease inhibitors (Roche tablet 1836153) (8 µL TBS/mg tissue). In brief, samples were gently sonicated (1 cycle of 35s, at 35% duty cycle and output 4 in a *Dynatech* Sonic Dismembrator ARTEK 300 with smallest tip), centrifuged at 100,000g for 1h at 4ºC, and the collected supernatant was labeled as *extracellular protein TBS fraction*. Subsequently, the insoluble pellet was dissolved to the same volume and rehomogenized in cold TBS-1% Triton X-100 solution supplemented with protease inhibitors, and centrifuged again; the supernatant was labeled as *intracellular protein 1% Triton fraction*. The Triton X-100 insoluble pellet was dissolved then to the same volume and rehomogenized in 2% SDS solution supplemented with protease inhibitors, and centrifuged again; the supernatant was labelled as *membrane-associated protein 2% SDS fraction*. Insoluble material was finally sonicated in 70% formic acid solution in water, centrifuged, and the supernatant was dried overnight using a vacuum concentrator (Savant SpeedVac® concentrator). The final protein extract was resuspended in 40 µL of DMSO. The total protein concentration of each soluble sample was determined by the BCA assay (Thermo Scientific, Rockford, IL). All of the fractions were aliquoted and stored at -80 ºC until its use.

Immunoblotting

Samples were heated in 2X Bicine loading buffer for 5 min at 95ºC and resolved by Bicine/BisTris SDS-PAGE system under reducing conditions and in the presence of Urea 8 M[50]. Proteins were transferred to PVDF membranes (Immobilon P^{sq} membrane,

Millipore) boiled for 7 min in PBS and blocked with Tween®20-Phosphate-Buffered Saline (T-PBS) containing 5% non-fat milk for 1h at 20ºC. After overnight incubation at 4ºC with primary antibody 6E10 (monoclonal antibody which recognizes all forms of Aβ, Signet Labs) at a dilution of 1:350, blots were washed in T-PBS for 20 min and incubated for 1h at 20ºC with HRP-conjugated secondary antibody (Bio-Rad) at a dilution of 1:2500. Blots were developed with ECL detection system (Supersignal Pico Western system, Pierce). Quantification of each band was performed by densitometry and reference to linear standards of synthetic human $A\beta_{1-42}$ peptide.

Clusterin and apoE ELISA

Murine ELISA kits were purchased from USCN Life Science Inc. and used as recommended by the supplier.

Statistics

Statistical analyses were performed using SPSS 12.0 software. In the behavioral studies, results are expressed as means ± SEM. The effects of the factors genotype (G), treatment (T) and time (t), as well as their interaction effects (GxT and txGxT), were analyzed with Repeated Measures ANOVA (RMA) and ANOVA with post-hoc Duncan or Student-*t*-test for independent samples. To analyze differences between two related samples, Paired *t*-test was used instead. The relationship between latencies and search strategies in the maze were investigated using Pearson correlation. In the molecular studies, results are expressed as mean ± SD and the Mann Whitney U test was used for assessing statistical differences. Statistical significance was always considered at $p<0.05$.

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The authors state that “there are no potential conflicts of interest in our submission”.

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LEGENDS TO TABLES

Table 1. BPSD-like behaviors in female 3xTg-AD mice at 5 months of age. Statistics: Student *t*-test, **p*<0.05, ****p*<0.001 *vs* NTg mice.

Table 2. Searching strategies in the probe trial. Incidence of animals exhibiting a certain search strategy expressed as percentage.

LEGENDS TO FIGURES

Figure 1. Triple-transgenic 3xTg-AD mice exhibited anxious-like behavior in the open field test before the treatment. Results are expressed as means ± SEM. The time course (left panels) of horizontal locomotor activity **(A)** and vertical locomotor activity **(B)** in the open field test shows that the 3xTg-AD mice exhibited reduced locomotor activity from the beginning to the end of test. The accumulated counts (right panels) indicated that at 5 months of age, the 3xTg-AD presented BPSD-like symptoms, in this case anxiety to a new open and illuminated environment, which implied a reduction of about 50% in their total horizontal **(A)** and vertical activity **(B)** as compared to the NTg animals. Statistics: Student's *t*-test * Indicates significant differences between 3xTg-AD (dark circles or bars, n=15) and NTg animals (light circles or bars, n=15), $p < 0.05$.

Figure 2. Effects of ScFv-h3D6 on learning and memory in the cue learning and the place task in the Morris water maze. Results are expressed as means ± SEM. The left panels illustrated the day-by-day long-term memory results obtained as mean latency **(A)** to reach the platform, the mean distance covered **(B)** and the swimming speed **(C)** during the cue learning (V) and the place task (PT). The right panels depict the results obtained in each trial of the test which involve long- and short-term learning and memory. As shown by the statistics, time (t), genotype (G) and treatment (T) effects were found in both long and short-term learning and memory results ***, p<0.001, ** p<0.01, * p<0.05, Post-hoc Duncan's test, * p<0.05 non-treated 3xTg-AD mice *vs* all the other groups, [a] p<0.05 *vs* the non-treated animals of the same genotype, [b] p<0.05 *vs* the other genotype with the same treatment conditions, [c] p<0.05, *vs* the non-treated 3xTg-AD mice.

Figure 3. Effects of ScFv-h3D6 in the probe trial in the Morris water maze. Results are expressed as means ± SEM. The swimming speed **(A)** was significantly increased in 3xTg-AD as compared to NTg mice and the treatment with scFv-h3D6 reversed this effect to normal swimming speed. The animals did not differ in the number of annulus crossings **(B).** Genotype per treatment (GxT) effects were found in the preferences shown in the different quadrants of the maze: P (trained quadrant were the platform was previously located), AL (adjacent left), O (opposed) and AR (adjacent right) during the free swim trial in both the time **(C)** and the distance covered **(D)**. A preference for the opposed quadrant was found in the 3xTg-AD mice but the treatment reduced its statistical significance D. Annulus crossings (number). Statistics: Between groups, ANOVA, Post-hoc Duncan's or Student *t*-tests, * $p<0.05$ *vs* the non-treated NTg mice; [a] $p<0.05$ *vs* the non-treated 3xTg-AD mice. Within groups, paired-t-test, [bbb], $p<0.001$ and [b] $p<0.05$ O *vs* P; [c] $p<0.05$ O *vs* AL.

Figure 4. Trajectories of the animals during the probe trial. The figure illustrates the navigation trajectories of all the animals during the probe trial which are thereafter (see Table 2) used to quantify the search strategy according to Lang *et al.*, 2003[49].

Figure 5. Immunoblotting analysis of the soluble Aβ-amyloid oligomers from extracellular extracts of 5 month-old NTg and 3xTg-AD mice. **(A)** Extracellular extracts from several brain subregions (HC, hippocampus; CX, cortex; OB, olfactory bulb and CR, cerebellum), from non-transgenic (NTg) and triple transgenic (3xTg-AD) mice I.P. treated with 85 μg of scFv-h3D6 (+) and I.P. treated with PBS (-), were analyzed. Profiles for NTg mice did not change upon scFv-h3D6 treatment. The profiles for 3xTg-AD mice of extracellular soluble Aβ oligomers in CX and OB showed a clear

decrease of the dodecameric, nonameric, hexameric and trimeric Aβ-species upon treatment (squared), while in HC and CR remained the same. Arrows indicate respective migration position of monomers (1-mer), trimers (3-mer), hexamers (6-mer), nonamers (9-mer), dodecamers (12-mer) and sAPPα (secreted form of APP that has been cleaved by α-secretase). Synthetic human $A\beta_{1-42}$ peptide ($hA\beta_{42}$) was used as a positive control (left lanes). Total protein applied to each lane was 45 µg. Blots were normalized by β-actin concentration. **(B)** Bar diagram showing the mean ± SD of the quantification of the bands from three experiments.

Figure 6. Immunoblotting analysis of the soluble Aβ-amyloid oligomers from intracellular and membrane-associated protein extracts of 5 month-old 3xTg-AD mice. Intracellular protein fractions **(A)** and membrane-associated protein fractions **(B)**, from several brain subregions (HC, hippocampus; CX, cortex; OB, olfactory bulb and CR, cerebellum), from triple transgenic (3xTg-AD) mice I.P. treated with 85 µg of ScFv-h3D6 (+) and I.P. treated with PBS (-), were analyzed. Only sAPPα, dodecamer and trimer Aβ-species in the intracellular fraction and sAPPα and nonamer Aβ-species in the membrane-associated fractions, were detected. But the profile pattern of the oligomeric species upon scFv-h3D6 treatment did not change. NTg groups rendered similar results (not shown). Arrows indicate respective migration position of monomers (1-mer), trimers (3-mer), nonamers (9-mer), dodecamers (12-mer) and sAPPα (secreted form of APP that has been cleaved by α-secretase). Synthetic human $A\beta_{1-42}$ peptide ($hA\beta_{42}$) was used as a positive control (left lanes). Total protein applied to each lane was 45 µg.

Figure 7. Clusterin (A) and apoE (B)concentrations in TBS extracts determined by ELISA. Mean ± SD, *n*=5, Mann-Whitney U test, **p*<0.05 *vs* untreated NTg mice. $^{\#}p$<0.05 *vs* untreated 3xTg-AD mice.

FIGURE 1

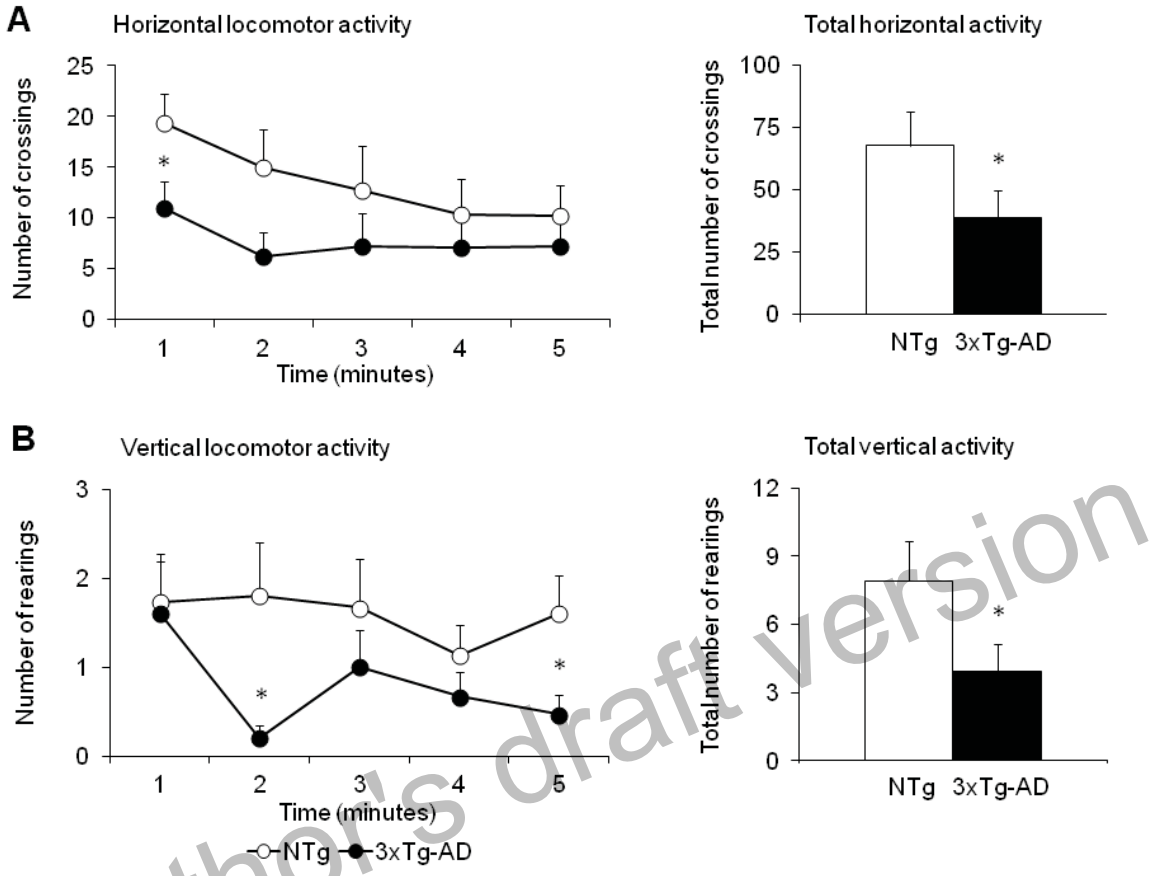


FIGURE 2

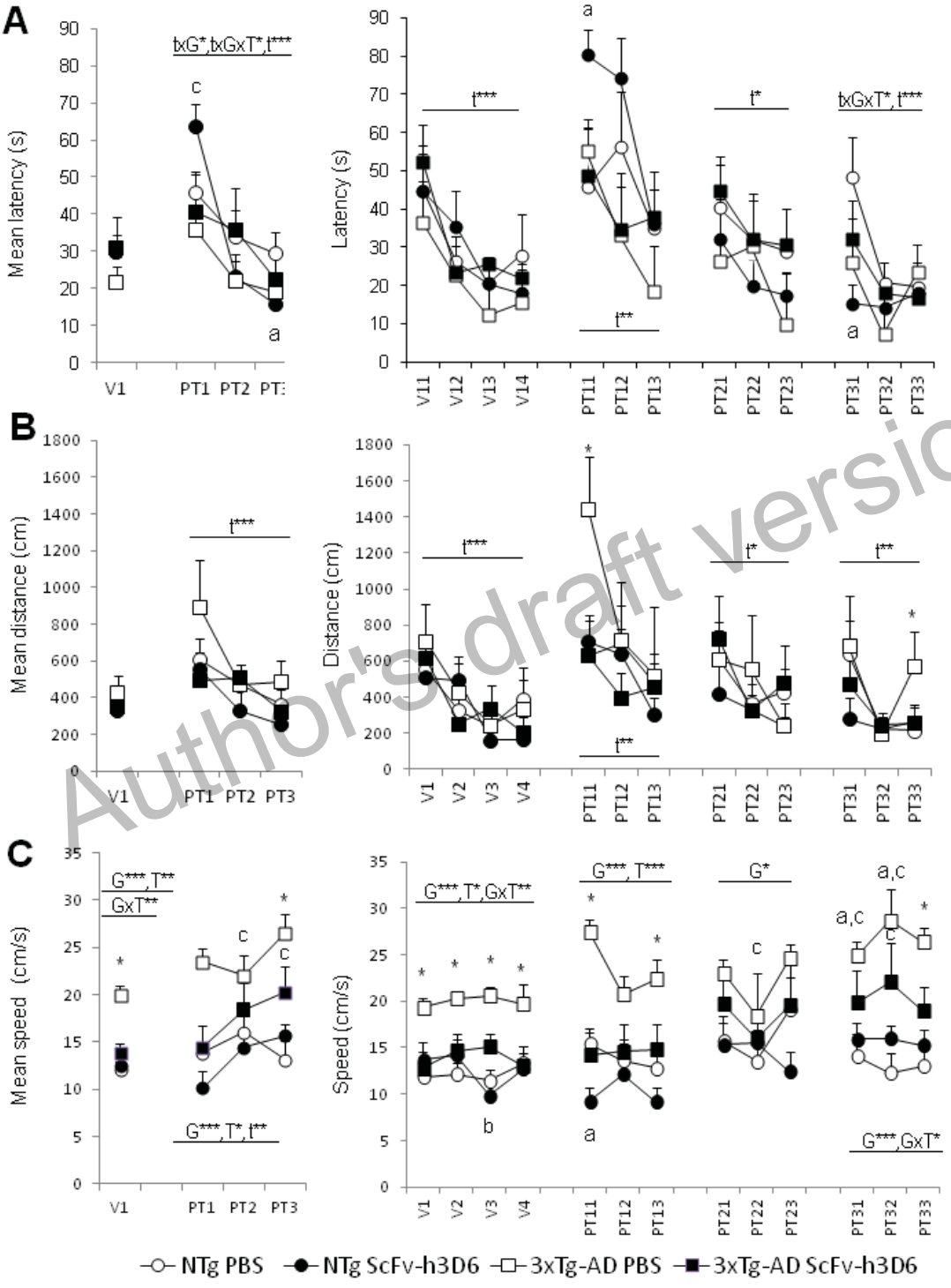


FIGURE 3

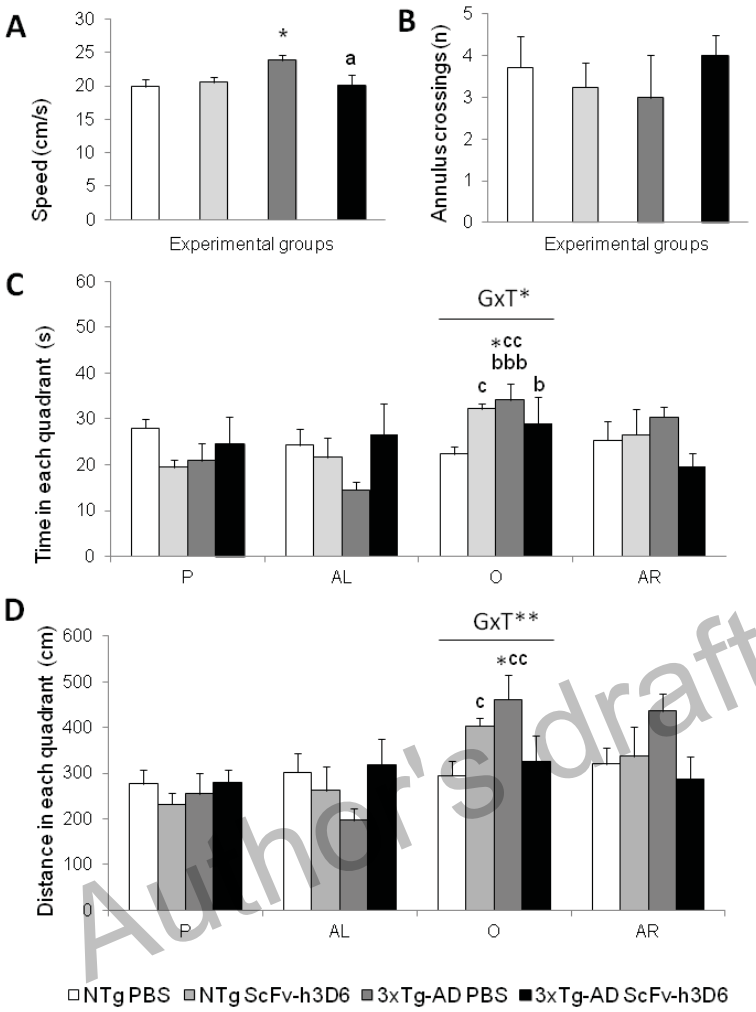


FIGURE 4

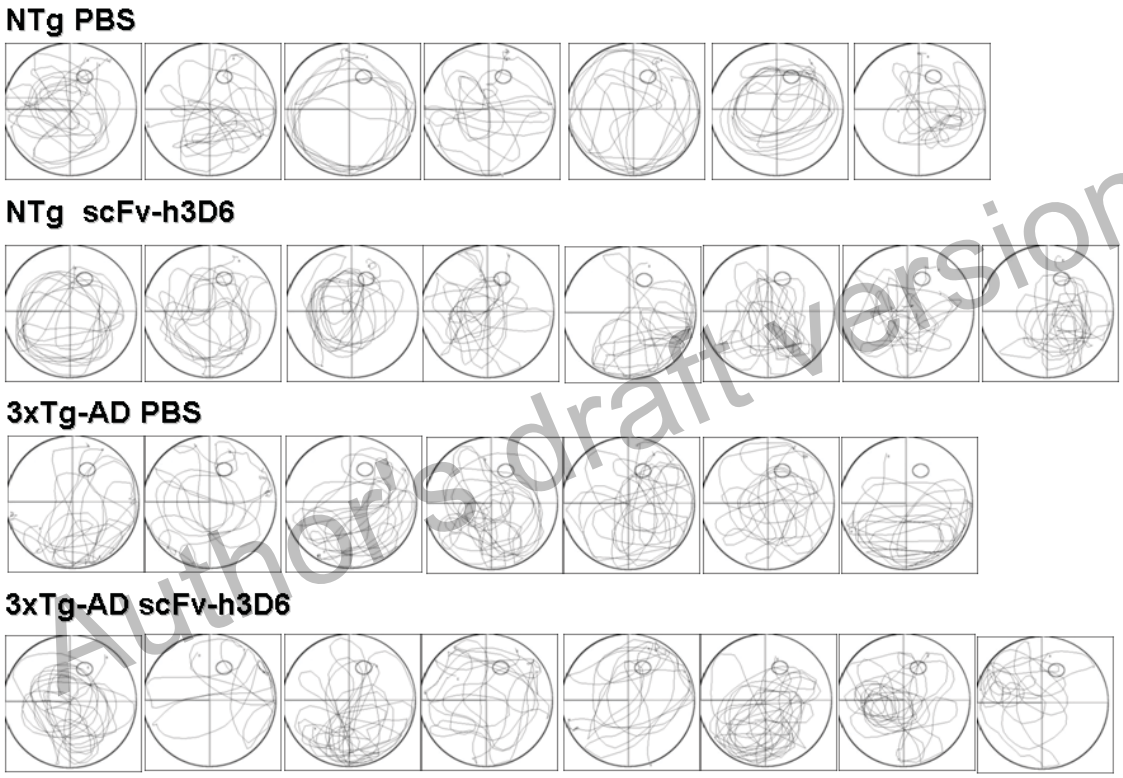
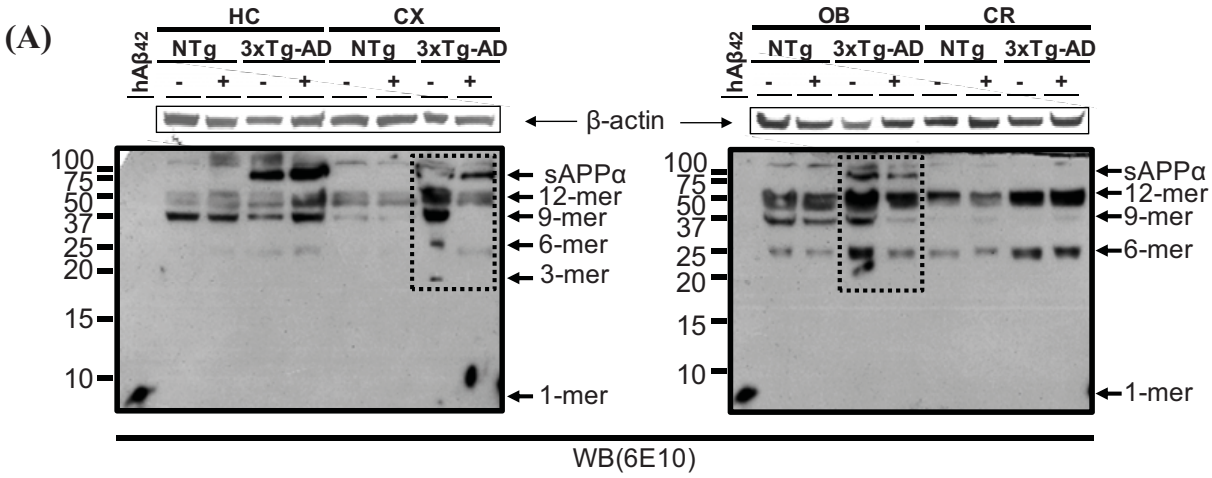


FIGURE 5



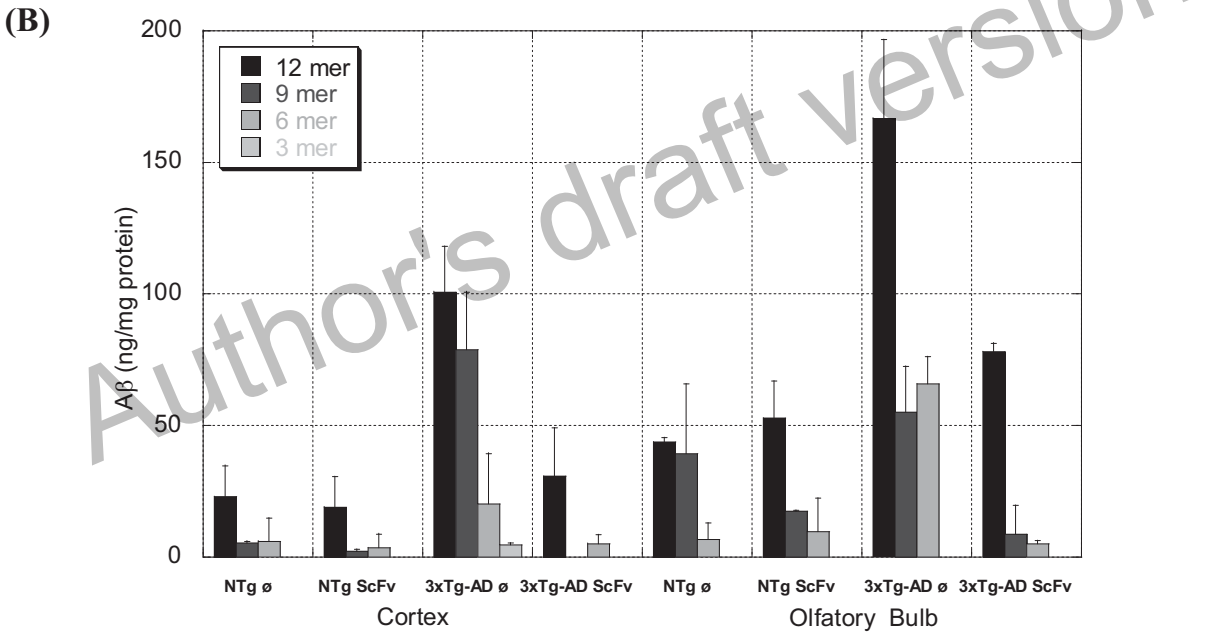
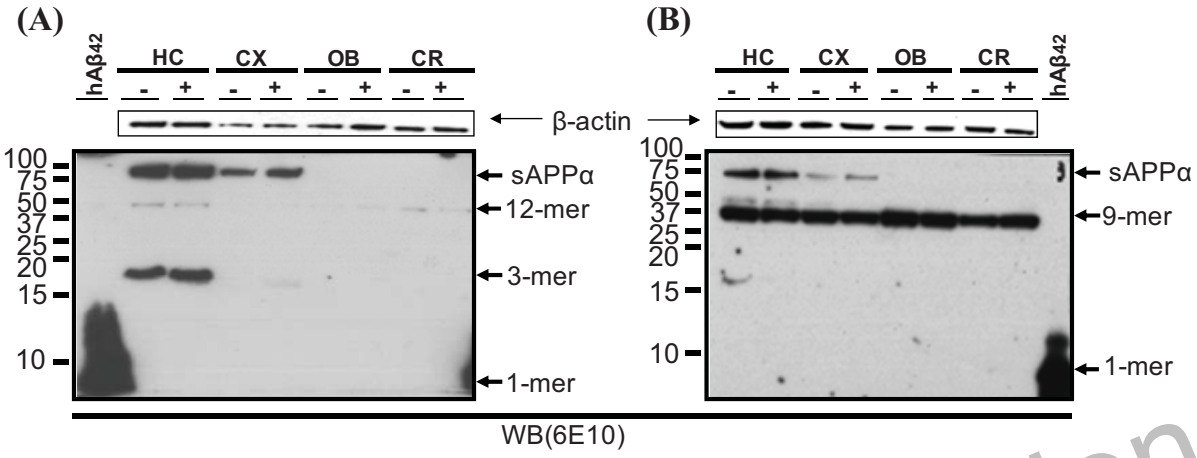


FIGURE 6



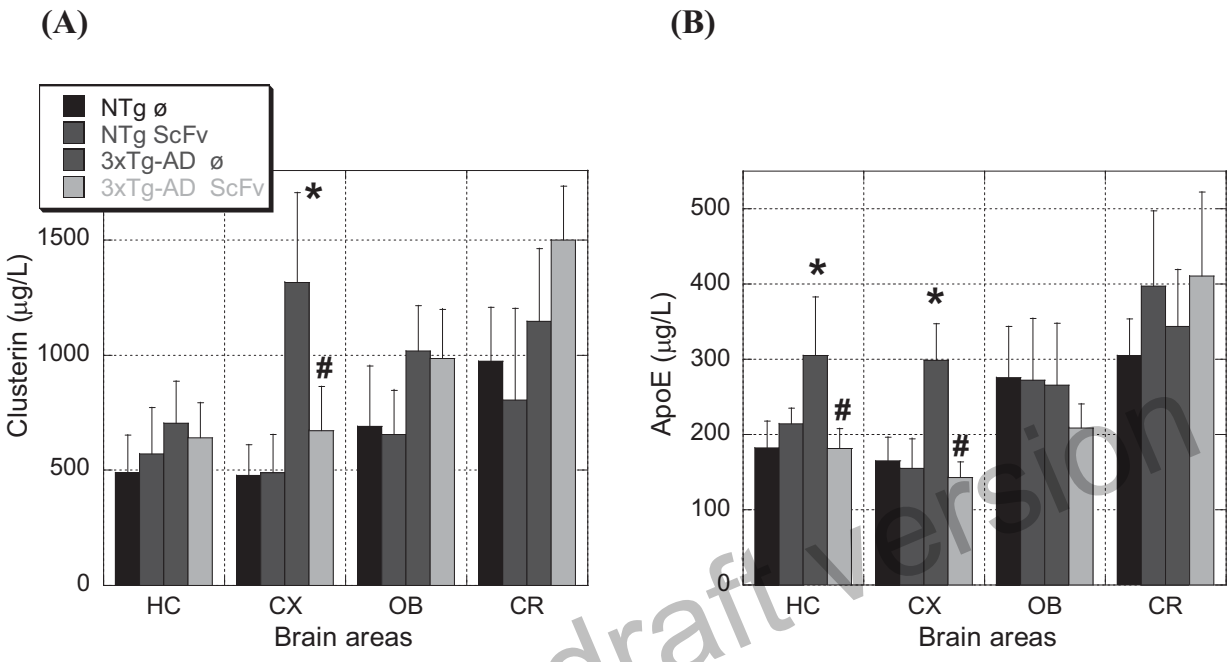


Table 1

	NTg *n*= 15	3xTg-AD *n*=15	Statistics
Corner test			
Locomotor activity			
Horizontal activity (corners, *n*)	**6.87 ± 0.68**	**9.13 ± 0.65**	*
Vertical activity (rearings, *n*)	**1.80 ± 0.46**	**3.00 ± 0.53**	*
Vertical activity (latency, s)	17.80 ± 2.60	15.20 ± 2.40	*n.s.*
Open-field test			
Freezing and thigmotaxis			
Initial freezing (latency, s)	**1.73 ± 0.43**	**3.53 ± 0.26**	***
Exit of the center (latency, s)	**5.47 ± 0.62**	**29.27 ± 10.52**	*
Entrance to periphery (latency, s)	**16.67 ± 3.15**	**79.47 ± 28.56**	*
Locomotor activity			
Horizontal activity (crossings, *n*)	**See Fig. 1a**		*
Vertical activity (rearings, *n*)	**See Fig. 1b**		*
Vertical activity (latency, s)	85.53 ± 25.00	154.33 ± 34.09	*n.s.*
Self-grooming behavior			
Self-grooming (incidence, *n*/total)	7/15	6/15	*n.s.*
Self-grooming (latency, s)	227.00 ± 85.64	259.27 ± 64.12	*n.s.*
Self-grooming (*n*)	0.67 ± 0.21	0.47 ± 0.17	*n.s.*
Self-grooming (duration, s)	2.33 ± 0.74	1.87 ± 0.71	*n.s.*
Other emotional behaviors			
Defecation boli (*n*)	2.93 ± 0.36	3.93 ± 0.50	*n.s.*
Urination (incidence, , *n*/total)	3/15	5/15	*n.s.*

Table 2

	NTg PBS *n*=7	NTg ScFv-h3D6 *n*=8	3xTg-AD PBS *n*=7	3xTg-AD ScFv-h3D6 *n*=8
Non-goal directed strategies				
Floating	0	0	1.10	0
Wall hugging	2.20	0	0	0
Chaining	1.10	0	**13.83**	**6.97**
Random swimming	27.29	21.63	22.71	30.77
Goal-directed strategies				
Scanning	47.16	47.52	41.85	45.27
Focal searching	**13.37**	**8.97**	5.77	3.04
Direct searching	**8.88**	**21.88**	14.74	13.94